Proteinase-Activated Receptor-2 (PAR_2): A Tumor Suppressor in Skin Carcinogenesis

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The proteinase-activated receptor PAR_2 has been demonstrated to modulate tumor growth, invasion and metastasis in various tissues. However, the role of PAR_2 in cutaneous cancerogenesis is still unknown. Here we could show a protective role of PAR_2 in the development of epidermal skin tumors: we established a mouse skin tumor model using chemically induced carcinogenesis. Tumors started to appear after eight weeks. After 13 weeks, PAR_2-deficient mice showed a significantly increased number of skin tumors (14 per animal on the average) in contrast to the wild type (eight tumors per mouse). Analysis of possible signal transduction pathways activated upon PAR_2 stimulation in HaCaT keratinocytes showed an involvement of extracellular signal-regulated kinase 1/2 and profound epidermal growth factor receptor transactivation, leading to secretion of the tumor-suppressing factor transforming growth factor-β1. Thus, our results provide early experimental evidence for a tumor-protective role of PAR_2.

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INTRODUCTION

Several observations suggest an important role of serine proteinases in regulating skin homeostasis, dermo-epidermal barrier function, cell differentiation, and tumor growth (reviewed in Ossovskaya and Bunnett, 2004). Recent findings clearly indicate that some serine proteinases, apart from their function to activate proteolytic enzyme cascades or to degrade proteins, are known to act as signal molecules via specific cleavage of certain seven-transmembrane domain G-protein-coupled receptors, the proteinase-activated receptors (PARs). So far, four members of this receptor class are known. PAR_1, PAR_3, and PAR_4 are activated by thrombin, whereas PAR_2 is stimulated by several trypsin-like enzymes including trypsin and mast-cell tryptase (reviewed in Steinhoff *et al.*, 2005). In addition, recent results show that PAR_1 can be also activated by matrix metalloproteinase-1 (MMP-1) (Boire *et al.*, 2005).

PAR_2 was mostly reported to play a growth-promoting role in tumors derived from numerous tissues (Darmoul *et al.*, 2004; Ge *et al.*, 2004; Hjortoe *et al.*, 2004; Jikuhara *et al.*, 2004; Shi *et al.*, 2004; Shimamoto *et al.*, 2004). In the skin, the receptor is strongly expressed by human keratinocytes (Santulli *et al.*, 1995; Derian *et al.*, 1997; Hou *et al.*, 1998; Steinhoff *et al.*, 1999; Algermissen *et al.*, 2000) and appears to play an important role in cutaneous homeostasis and tissue repair (reviewed in Rattenholl and Steinhoff, 2003). PAR_2 agonists increase $[Ca^{2+}]_i$ (Santulli *et al.*, 1995), presumably resulting in inhibition of growth and differentiation of keratinocytes (Derian *et al.*, 1997). *In vivo*, PAR_2 on epidermal keratinocytes could be activated by an autocrine mechanism via keratinocyte-derived trypsinogen IV that was reported to activate PAR_2 and PAR_4 (Cottrell *et al.*, 2004).

Although PAR_2 is highly expressed by human keratinocytes, its role in human skin carcinogenesis is still unknown. Therefore the aim of this study was (a) to investigate the development of chemically induced skin tumors in PAR_2-deficient mice compared to the wild-type, (b) to examine potential signal transduction pathways involved in PAR_2-mediated suppression of skin cancerogenesis using the keratinocyte cell line HaCaT, and (c) to identify possible tumor suppressing factors released after stimulation of PAR_2.

RESULTS

Increased skin carcinogenesis in PAR_2-deficient mice

To investigate a possible tumor suppressing effect of PAR_2 in epidermal keratinocytes, chemically induced skin

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Abbreviations: BCC, basal-cell carcinoma; CDK, cyclin-dependent kinase; DMBA, 7,12-dimethylbenz[a]anthracene; EGF(R), epidermal growth factor (receptor); ERK , extracellular signal-regulated kinase ; HB-EGF, heparin binding-epidermal growth factor; K6/10, keratin 6/10; MAPK, mitogen-activated protein kinase; MEK1, mitogen-activated protein kinase kinase-1; SCC, squamous-cell carcinoma; SSC, saline-sodium citrate buffer; PMA, phorbol myristate acetate (12-O-tetradecanoylphorbol-13-acetate); PAR, proteinase-activated receptor; TACE, TNF-α-converting enzyme; TAPI-1, TNF-α protease inhibitor-1; TGF-α/β1, transforming growth factor-α/β1; TNF-α, tumor necrosis factor-α; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; WT, wild type

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carcinogenesis was monitored for 13 weeks in PAR_2-deficient and wild-type mice. Whereas only few papillomas could be observed in wild-type animals, PAR_2-deficient mice had developed numerous epidermal skin tumors. A quantitative analysis of tumor numbers is shown in Figure 1. In both groups, tumors started to appear after seven weeks. Two weeks later, all mice had developed papillomas (Figure 1a). However, after 13 weeks, the PAR_2-deficient animals had formed a markedly enhanced number of tumors as compared to the wild-type animals (Figure 1b): these PAR_2-deficient mice exhibited more than 14 tumors per mouse on the average. In contrast, wild-type animals had formed only eight epidermal skin tumors (Figure 1c). Mere application of the vehicle (acetone/olive oil mixture) after 7,12-dimethylbenz-[a]anthracene (DMBA) treatment did not lead to macroscopic tumor formation (data not shown).

Histological analysis of tumor sections in both groups showed that all tumors were benign papillomas, characterized by marked papillary hyperplasia and thickening of the epidermis in contrast to the unaffected skin.

Analysis of epidermal cell differentiation in papillomas of PAR_2-deficient mice

As skin carcinogenesis is associated with keratinocyte transformation, the differentiation of epidermal keratinocytes in papillomas of wild-type and PAR_2-deficient mice was analyzed by immunohistochemistry using representative marker proteins. Keratin 10 (K10), an early marker for keratinocyte differentiation (Huitfeldt *et al.*, 1991), was expressed in suprabasal keratinocytes to the same extent in normal skin sections of both genotypes (Figure 2a, b). Moreover, K10 immunoreactivity was reduced to a comparable degree in papillomas derived from both wild-type and PAR_2-deficient animals: all tumors examined exhibited regions with a downregulation or even absence of K10. Two examples are displayed in Figure 2c, d. However, the number of K10-positive basal keratinocytes was slightly enhanced in wild-type papillomas (Figure 2i). Loricrin, a marker for late terminal keratinocyte differentiation (Mehrel *et al.*, 1990) could be found in the granular layers of normal skin (Figure 2e, f) and in tumors of PAR_2-deficient mice (Figure 2g, h). No differences in loricrin expression were observed in wild-type and PAR_2-deficient papillomas (Figure 2j).

Investigation of tumor angiogenesis and infiltration with inflammatory cells in papillomas

Next, the presence of blood vessels in the tumors was analyzed by immunohistochemistry using the marker CD31 (platelet/endothelial cell adhesion molecule-1). As expected, numerous blood vessels could be found in papillomas of both genotypes (Figure 3a, b). However, angiogenesis seemed to be slightly upregulated in PAR_2-deficient papillomas (Figure 3c) compared to wild-type tumors: when counting the large vessels ($\geqslant 30\,\mu$m in diameter), PAR_2-deficient tumors contained 85 vessels per mm^2 subbasal area compared to only 51 in the wild-type controls (Figure 3c).

To assess the infiltration of papillomas in both genotypes by inflammatory cells (macrophages and granulocytes), the marker CD11b (Mac-1a) was used. No difference could be found in the tumors of both genotypes: all papillomas analyzed showed a comparable degree of infiltration with CD11b-positive cells (Figure 3d). Most of these cells could be detected in the mesenchyme. However, some of them could

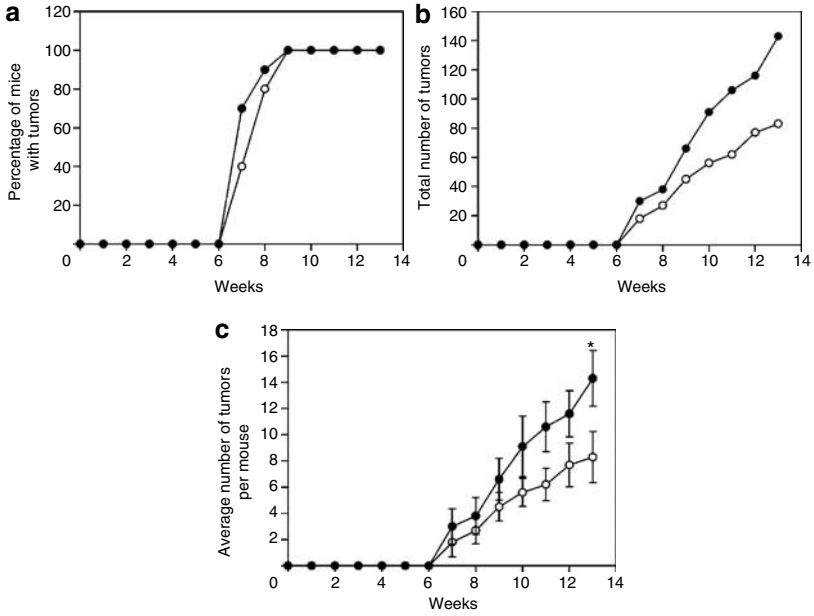

Figure 1. Chemically induced tumor formation is promoted in PAR_2-deficient mice. (**a**) Tumors started to develop after seven weeks. After nine weeks, all animals were carrying at least one papilloma. (**b**, **c**) After 13 weeks, PAR_2-deficient mice had developed a total of 143 papillomas (14 on the average per animal) compared to only 83 (mean: eight per mouse) in the wild-type controls ($n = 10$ for each group; $^*P \leqslant 0.05$).

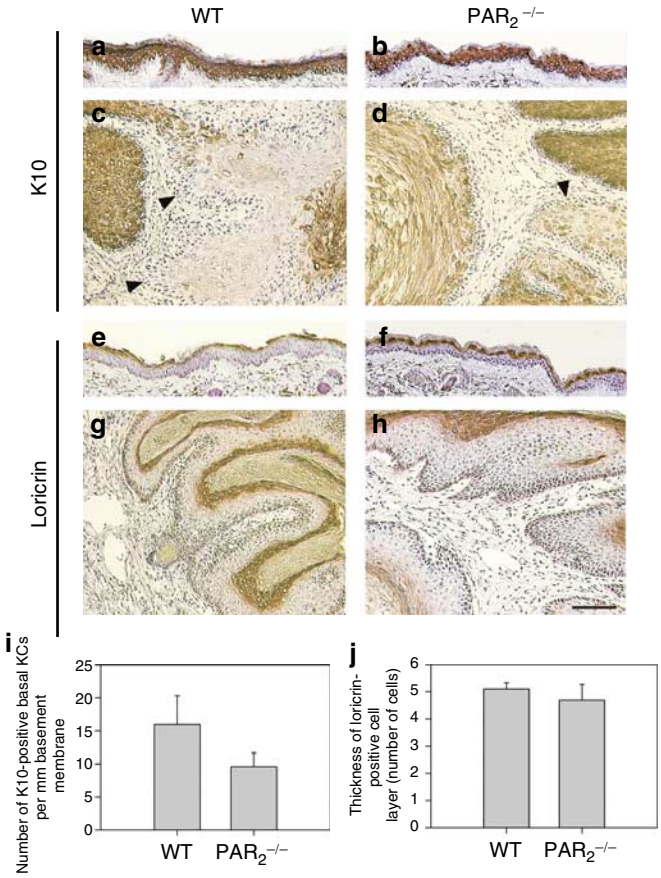

Figure 2. Analysis of keratinocyte differentiation in mouse papillomas. (**a**, **b**) Immunoreactivity for the differentiation marker K10 in normal skin. (**c**, **d**) Staining for K10 in papillomas. Tumors of both genotypes show regions with absent or low immunoreactivity for K10 (arrowheads). (**e**, **f**) Expression of loricrin, a marker for late terminal keratinocyte differentiation, in normal skin. (**g**, **h**) Staining for loricrin in mouse tumors. (**i**) Quantitative analysis of K10 expression in basal keratinocytes. WT papillomas contain a somewhat larger number of K10-positive basal cells ($n = 7$ WT papillomas from six individual mice; $n = 9$ PAR_2-deficient papillomas obtained from eight animals). (**j**) Comparable thickness of the loricrin-positive cell layer in tumors of both genotypes ($n = 7$ WT papillomas from six mice; $n = 10$ PAR_2-deficient tumors obtained from eight animals). Bar = 100 μm.

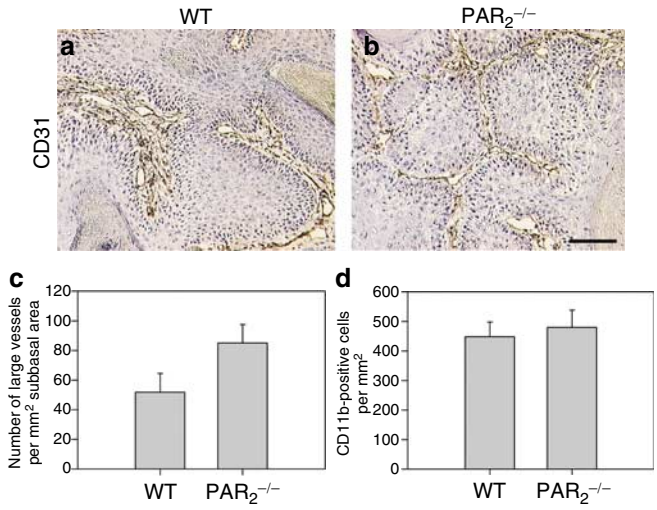

Figure 3. Analysis of angiogenesis and infiltration with inflammatory cells in mouse papillomas. Immunoreactivity for the endothelial cell marker CD31 in a (**a**) WT and (**b**) $PAR_2^{-/-}$ papilloma. Bar = 100 μm. (**c**) Only vessels with a diameter ≥ 30 μm were taken for the quantitative analysis of blood vessel formation: PAR_2-deficient mouse papillomas contain a slightly larger number of large blood vessels compared to the wild-type ($n = 7$ WT tumors from seven animals; $n = 10$ PAR_2-deficient papillomas from eight mice). (**d**) Comparable infiltration with CD11b-positive inflammatory cells in tumors of both genotypes ($n = 10$ WT tumors from seven individual mice; $n = 8$ PAR_2-deficient papillomas from six animals).

also be found in the epidermis, in the vicinity of the basement membrane (not shown).

Apoptosis is not decreased in papillomas of PAR_2-deficient mice

Paraffin sections obtained from $PAR_2^{-/-}$ and wild-type papillomas were screened for keratinocytes that underwent apoptosis. Apoptotic cells were visualized by TUNEL staining (Schön *et al*, 2004). Some apoptotic cells could be observed at the border to the granular layer in both genotypes (Figure 4a, b). The apoptotic rate was low in both groups.

Activation of PAR_2 leads to extracellular signal-regulated kinase 1/2 phosphorylation in cultured HaCaT keratinocytes

Next, possible signal transduction pathways activated upon PAR_2 stimulation in HaCaT keratinocytes, an immortalized human keratinocyte cell line, were studied. HaCaT keratinocytes produce PAR_1, PAR_2, and PAR_4 mRNAs (unpublished results). Cells were serum-starved for 24 hours before the addition of activating peptide (SLIGKV-NH_2). After certain incubation times, cells were lysed and probed for phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) by immunoblotting. Indeed, as soon as 5 minutes after addition of PAR_2-AP, activation of ERK1/2 could be observed (Figure 5a). Phosphorylation decreased after 30 minutes but could be detected up to 4 hours after stimulation of PAR_2. In contrast to the positive control obtained with sorbitol, no activation of the p38 pathway after stimulation of HaCaT cells with the PAR_2-activating peptide could be observed (Figure 5b).

PAR_2 stimulation causes EGFR transactivation in HaCaT keratinocytes

PAR_2-mediated ERK1/2 phosphorylation in HaCaT keratinocytes involved transactivation of the EGFR because ERK1/2 activation was completely abolished in the presence of the specific EGFR tyrosine kinase inhibitor PD153035 (Figure 6a). Next, we seeked to inhibit PAR_2-dependent EGFR transactivation on HaCaT keratinocytes using several metalloproteinase inhibitors: MMP inhibitor II is a potent inhibitor of MMP-1, -3, -7, and –9; MMP inhibitor III inhibits MMP-1, -2, -3, -7, and -13; *o*-phenanthroline is an unspecific metalloproteinase inhibitor that acts via removal of the zinc ion from the catalytic site; tumor necrosis factor-α protease inhibitor-1 (TAPI-1) is an inhibitor for the membrane-bound

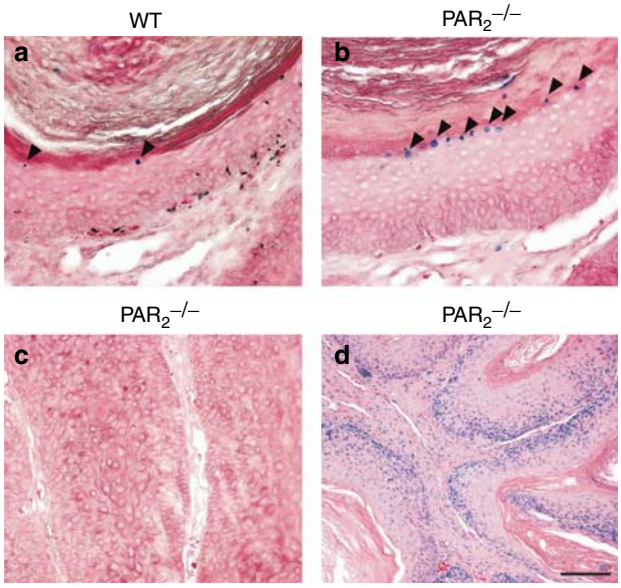

Figure 4. Apoptotic keratinocytes in mouse papillomas (TUNEL assay). Both WT and PAR_2-deficient papillomas exhibit a comparable, low rate of apoptosis. (**a**) WT papilloma. Few apoptotic cells can be found adjacent to the granular layer (**b**) PAR_2-deficient tumor. Some keratinocytes undergoing apoptosis can be detected. (**c**) Negative control ($PAR_2^{-/-}$ papilloma). (**d**) Positive control (PAR_2-deficient papilloma). Arrowheads denote apoptotic cells. (**a**–**c**) Bar = 50 μm. (**d**) Bar = 100 μm.

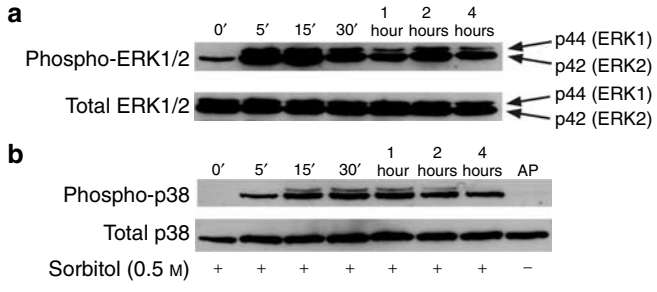

Figure 5. Activation of PAR_2 leads to rapid phosphorylation of ERK1/2 but not p38. (**a**) Cells were stimulated with 10^{-4} M PAR_2-AP for up to four hours. As soon as five minutes after addition of the peptide, phosphorylation of ERK1/2 can be observed. ERK1/2 activation diminishes after 30 minutes. (**b**) Stimulation of HaCaT cells with 0.5 M sorbitol leads to fast phosphorylation of p38. In contrast, p38 is not phosphorylated after activation of PAR_2 with the activating peptide (AP). Shown are representative immunoblots from three independent experiments.

metalloproteinase tumor necrosis factor-α converting enzyme (TACE/ADAM17) which belongs to the ADAM (a disintegrin and metalloproteinase) family of sheddases (Vincent *et al.*, 2001). Signal inhibition was again assessed by analysis of ERK1/2 phosphorylation. Indeed, all inhibitors strongly inhibited ERK1/2 phosphorylation, indicating that tumor necrosis factor-α converting enzyme and maybe other sheddases provoke shedding of EGFR ligands after activation of PAR_2 (Figure 6b, c).

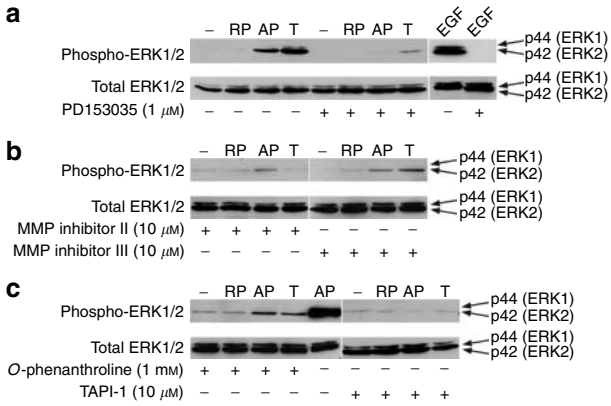

Figure 6. Stimulation of PAR_2 is followed by EGFR transacivation. (**a**) PAR_2-induced ERK1/2 phosphorylation is nearly completely abolished in the presence of the EGFR kinase inhibitor PD153035. (**b**, **c**) PAR_2-triggered EGFR transactivation in HaCaT keratinocytes is mediated by metalloproteinase activity: phosphorylation of ERK1/2 is inhibited considerably in the presence of various metalloproteinase inhibitors. (**c**) A positive control is loaded on lane five. The immunoblots shown are a representative of three independent experiments. –, negative control; RP, reverse peptide for PAR_2 (10^{-4} M); AP, activating peptide for PAR_2 (10^{-4} M); T, trypsin (10^{-7} M); EGF, epidermal growth factor (positive control, 50 ng/ml).

PAR_2 activation induces the release of the tumor suppressor TGF-β1 from HaCaT keratinocytes

To investigate further a possible production of tumor suppessing factors after EGFR transactivation on HaCaT keratinocytes, the secretion of TGF-β1 was assessed after stimulation with either trypsin or the PAR_2 agonist *trans*-cinnamoyl-LIGRLO-NH_2 (tc-AP) (Roy *et al.*, 1998), which is more stable compared to the other activating peptide used in this study (Figure 7). After 4 days of activation, the TGF-β1 concentration in the medium was increased about 6.1-fold in the presence of tc-AP, as compared to the unstimulated control. When HaCaT cells were stimulated with trypsin, the TGF-β1 content was increased 3.8-fold. Next, secretion of TGF-β1 was analyzed after inhibition of the ERK signal transduction pathway. Mitogen-activated protein kinase (MAPK) kinase-1 (MEK1) is a kinase upstream of ERK1/2. Indeed, preincubation of the HaCaT cells with the MEK1 inhibitor PD98059 led to a significant inhibition of TGF-β1 secretion in the presence of trypsin. Here, the TGF-β1 concentration in the medium reached almost the level of the negative control. Thus, TGF-β1 secretion mediated by trypsin was fully dependent on PAR_2-mediated EGFR transactivation. Interestingly, TGF-β1 secretion provoked by the agonist tc-AP was only insignificantly inhibited by the MEK1 inhibitor.

DISCUSSION

In this study, we demonstrate for the first time a role for PAR_2 as an inhibitor of the development in keratinocyte-derived skin tumors *in vivo*. In contrast, this receptor was mostly reported to promote proliferation of cells, for example astrocytes (Wang *et al.*, 2002) and various cancer cells

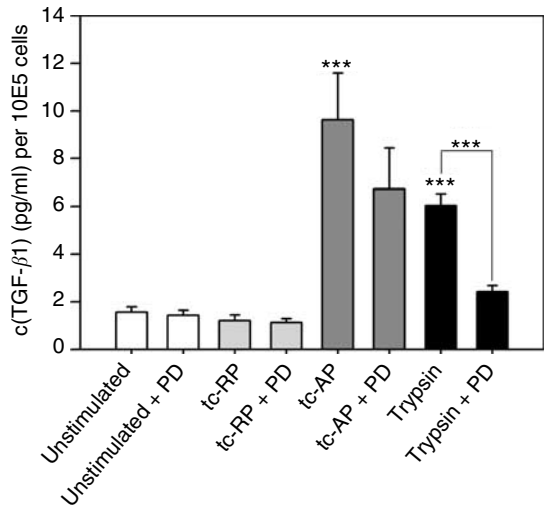

Figure 7. PAR_2 activation induces TGF-β1 secretion from HaCaT keratinocytes. After 96 hours, the TGF-β1 content in the medium was 6.1-fold higher in the presence of tc-AP, compared to the unstimulated control. In the presence of trypsin, the concentration was enhanced 3.8-fold. After addition of the MEK1 inhibitor PD98059, TGF-β1 secretion was significantly inhibited in the presence of trypsin. Interestingly, in the presence of tc-AP, TGF-β1 secretion was only slightly reduced. In the presence of the scrambled peptide tc-RP, no induction of TGF-β1 production could be observed. All experiments were carried out three times. Unst., unstimulated; tc-RP, tc-RP (10^{-4} M); tc-AP, tc-AP (10^{-4} M); Trypsin, trypsin (10^{-8} M); +PD, additional supplementation with PD98059 (10 μM). ***$P \leqslant 0.001$.

(Jikuhara *et al.*, 2004; Shi *et al.*, 2004; Shimamoto *et al.*, 2004; Wilson *et al.*, 2004). However, in agreement with our results, others found that trypsinogen expression was downregulated in esophageal squamous-cell carcinomas and certain gastric carcinomas (Yamashita *et al.*, 2003). Moreover, in these gastric carcinomas, PAR_2 expression was also markedly reduced. In addition, other studies suggested a role of PAR_2 as a negative regulator in human pancreatic tumor growth (Kaufmann *et al.*, 1998). Okamoto *et al.* (2001)reported that a human glioblastoma cell line produced both PAR_1 and PAR_2. In the presence of a PAR_2 agonist, cell proliferation was markedly inhibited, whereas a PAR_1 agonist was not able to exert this effect. In conclusion, these findings point to a tumor-suppressing role of PAR_2 only in certain tissues.

PAR_1 and PAR_2 modulate keratinocyte growth and differentiation in cultured human keratinocytes (Derian *et al.*, 1997; Algermissen *et al.*, 2000). Although PAR_1 induces growth and proliferation in primary human keratinocytes, PAR_2 inhibits proliferation and differentiation.

To elucidate further these notions, mice deficient in PAR_2 as well as wild-type controls were subjected to a two-stage chemical skin carcinogenesis standard protocol. Although both groups of animals showed similar latency periods, that is tumor growth was detectable after seven weeks in both groups, PAR_2-deficient mice indeed developed a significantly increased number of tumors as compared to the wild-type counterparts.

Epithelial skin carcinogenesis is associated with loss of keratinocyte differentiation (Jeon *et al.*, 2004). Comparison of early and late terminal keratinocyte differentiation by utilizing the marker K10 (Huitfeldt *et al.*, 1991) and loricrin (Mehrel *et al.*, 1990), respectively, showed that no differences in loricrin expression could be detected but a slight increase in K10-positive basal keratinocytes in wild-type papillomas. It is known that the expression of K10 in the basal layer of the epidermis inhibits cell proliferation and prevents skin tumorigenesis (Santos *et al.*, 2002). In contrast, loss of K10 leads to decreased tumor formation in mice due to increased keratinocyte turnover (Reichelt *et al.*, 2004). In this study, all papillomas investigated showed regions with a downregulation or even absence of K10 immunoreactivity in suprabasal epidermal layers.

Several groups have shown that inflammatory mast cells are able to affect angiogenesis in epidermal skin tumor development (Coussens *et al.*, 1999; Moore *et al.*, 1999). Immunohistochemical analysis of papillomas obtained from PAR_2-deficient and wild-type mice using the marker CD11b, which stains mast cells and other inflammatory cells, did not show any differences between $PAR_2^{-/-}$ and $PAR_2^{+/+}$ animals. In addition, immunoreactivity for CD31, a marker for blood vessels, in PAR_2-deficient and wild-type mice was analyzed. For a more convenient analysis, only large vessels with a diameter of at least 30 μm were counted. Papillomas from both $PAR_2^{-/-}$ and $PAR_2^{+/+}$ animals displayed a comparable number of large vessels. However, PAR_2-deficient tumors seemed to contain slightly more large blood vessels. Thus, angiogenesis might be enhanced in these papillomas.

Additional cell culture experiments using the cell line HaCaT were performed to analyze signal transduction pathways activated upon PAR_2 stimulation which could explain, at least in part, the antiproliferative effect of the receptor in keratinocytes. First, an involvement of the MAPKs ERK1/2 was found. In contrast, the p38 pathway was not turned on. The MAPK p38 was reported to be phosphorylated after PAR_2 activation in breast cancer cells (Liu and Mueller, 2006). ERK1/2 were also reported to be phosphorylated upon PAR_2 stimulation in the colon cancer cell line HT-29 (Darmoul *et al.*, 2004). However, receptor activation was associated with proliferation here. Nevertheless, our findings are in agreement with an earlier study demonstrating that in keratinocytes, stimulation of PAR_2 caused inhibition of cell growth, even in the presence of EGF and bovine pituitary extract (Derian *et al.*, 1997).

It is well known that numerous G-protein-coupled receptors, including PAR_1 and PAR_2, are able to transactivate the EGFR (Prenzel *et al.*, 1999; Sabri *et al.*, 2002; Darmoul *et al.*, 2004). Transactivation involves shedding of EGFR ligands (i.e. TGF-α or heparin-binding EGF) from the cell surface by metalloproteinases, subsequently activating EGFR in an autocrine/paracrine manner (Prenzel *et al.*, 1999). We found that PAR_2-mediated ERK1/2 phosphorylation in HaCaT keratinocytes was also fully dependent on EGFR transactivation, involving tumor necrosis factor-α converting enzyme and maybe other metalloproteinases.

To investigate further a possible secretion of tumor suppessing factors after PAR_2 activation in HaCaT keratinocytes, the secretion of TGF-β1 after stimulation with either trypsin or the PAR_2 agonist tc-AP was assessed. TGF-β1 is well known to be a potent inhibitor of keratinocyte proliferation (Dahler *et al.*, 2001; Yamasaki *et al.*, 2003). Indeed, TGF-β1 secretion was significantly stimulated by activation of PAR_2. Thus, we conclude that PAR_2 might exert its antiproliferative effects at least partly through TGF-β1 release via transactivation of EGFR (Figure 8). Moreover, in the presence of the ERK1/2 pathway inhibitor PD98059, trypsin-induced secretion of TGF-β1 was inhibited close to the level of the negative control. Interestingly, TGF-β1 production was only insignificantly reduced in the presence of PD98059 after stimulation with tc-AP. It might be possible that tc-AP does not selectively activate PAR_2 but one or several others, yet unidentified receptor(s) on HaCaT cells. Indeed, McGuire *et al.* (2002) found this also to be true in mouse vasculature. Hence, these findings can also be extended to human cells.

Together, our results clearly show an important role of PAR_2 as a tumor suppressor in the skin, possibly by regulating K10 expression, suppression of angiogenesis and stimulation of TGF-β1 secretion.

MATERIALS AND METHODS

Reagents

Chemicals were from Sigma Chemical Co. (Deisenhofen, Germany), unless otherwise stated. Human PAR_2-activating peptide SLIGKV-NH_2 (PAR_2-AP), the reverse peptide VKGILS-NH_2 (PAR_2-RP), *trans*-cinnamoyl-LIGRLO-NH_2 (tc-AP), and the correlating negative control *trans*-cinnamoyl-OLRGIL-NH_2 for the activation of PAR_2 were synthesized by the Peptide Synthesis Facility of the University of Calgary, Department of Biochemistry and Molecular Biology, Canada. EGFR kinase inhibitor PD153035, MMP inhibitors II and III, tumor necrosis factor-α protease inhibitor-1, and MEK1 inhibitor PD98059 were obtained from Calbiochem (Schwalbach, Germany). Human recombinant EGF was purchased from Peprotech (London, UK).

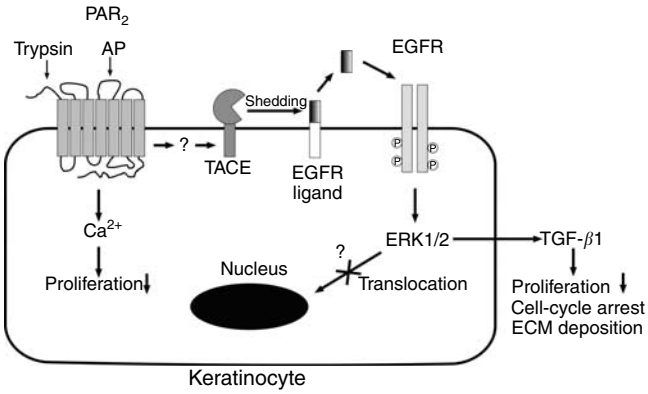

Figure 8. Schematic representation of PAR_2-mediated signaling in keratinocytes. PAR_2 is stimulated either by selective proteolytic cleavage or an activating peptide. This is followed by intracellular calcium mobilization and activation of tumor necrosis factor-α converting enzyme and maybe other metalloproteinases that are able to shed EGFR ligands from the cell surface. Activation of EGFR leads to phosphorylation of ERK1/2 and secretion of TGF-β1.

Mice

Studies were performed in male PAR_2-deficient ($PAR_2^{-/-}$) and wild-type ($PAR_2^{+/+}$) mice (genetic background: C57BL/6 strain) between 8 and 10 weeks of age when starting the carcinogenesis protocol. Animals were obtained from Charles River Laboratories (Toulouse, France). All animal experiments were approved by the Animal Protection Committee of the University Hospital of Münster.

Chemically induced carcinogenesis

A two-stage skin tumorigenesis protocol was employed, using DMBA as initiating agent and phorbol myristate acetate (12-*O*-tetradecanoylphorbol-13-acetate) as a promoter (Elmets *et al*, 1998). To this end, mice were shaved on the back and painted with 100 μg of DMBA (0.1 % (w/v) stock solution in a mixture of 75 % (v/v) acetone and 25 % (v/v) olive oil). In addition, 50 μg of DMBA was applied topically on one ear. Beginning 1 week later, 40 nmol (back) and 20 nmol (ear) phorbol myristate acetate (800 μM stock solution in acetone/olive oil) was applied biweekly to the sites that had been treated previously with DMBA. Mice were evaluated weekly for tumors.

Immunohistochemistry

Normal mouse skin and tumors for cryostat sections were snap-frozen in liquid nitrogen, embedded in OCT compound (Miles, UK) and sectioned. Decoration with primary antibodies (polyclonal rabbit anti-K10, anti-loricrin (all from Covance, Berkeley, CA, USA), monoclonal rat anti-CD11b (Chemicon, Chandlers Ford, UK), monoclonal rat anti-CD31 (Cymbus Biotechnology Ltd, Chandlers Ford, UK) (all dilutions 1:1,000 in 1% (w/v) bovine serum albumin in phosphate-buffered saline) was performed overnight at 4°C. Sections incubated with monoclonal rat antibodies were subsequently decorated with biotinylated rabbit anti-rat IgG for 1 hour at room temperature (RT; 1:1,000 in 1% (w/v) bovine serum albumin/phosphate-buffered saline; Vector Inc., Burlingame, CA, USA). Tissues were incubated with EnVision anti-rabbit/horseradish peroxidase-labeled polymer (DakoCytomation, Hamburg, Germany) for 1 hour at RT. Immunoreactions were visualized using the DAB peroxide substrate kit from DakoCytomation (Hamburg, Germany) according to the manufacturer's instructions. Nuclei were counterstained with Hematoxylin QS (Vector Inc.). Slides were mounted in Aquamount (Merck, Darmstadt, Germany) and analyzed with a DM LB microscope (Leica, Solms, Germany), equipped with a HV-C20M CCD camera (Hitachi, Rodgau, Germany) and Diskus 4.20 software (Carl H. Hilgers, Königswinter, Germany). For the quantification of immunohistochemical stainings, three representative pictures were taken from each papilloma analyzed. Quantification of immunoreactivity for K10 was taken out by measuring the number of K10-positive basal keratinocytes per mm basement membrane. Immunohistochemical analysis of loricrin was carried out by counting the loricrin-positive keratinocyte cell layers. Infiltration with CD11b-positive cells was quantified by counting the cells in a certain area. Angiogenesis was measured by determining the number of dermal blood vessels with a diameter of at least 30 μm per mm^2 subbasal area.

Apoptosis assay

Detection of apoptosis in paraffin sections of mouse papillomas by TUNEL assay was carried out as described (Schön *et al.*, 2004).

Cell extraction and immunoblotting

HaCaT cells (kindly provided by Dr. Petra Boukamp, German Cancer Research Center, Heidelberg, Germany) were cultivated in 10 cm Petri dishes. After 24 hours of serum deprivation, 10 μl dimethylsulfoxide or the desired inhibitors were added. After 15 minutes of incubation, cells were stimulated for 5 minutes in the presence of PAR_2-AP (10^{-4} M) or trypsin (10^{-7} M). Cells were extracted by direct lysis with 600 μl hot Laemmli buffer, scraped off, boiled for 8 minutes, and 10 μl of each extract were loaded onto a 10% (w/v) polyacrylamide gel. After electrophoresis, proteins were transferred to a BA85 nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Membranes were blocked with 5% (w/v) nonfat dry milk in tris-buffered saline containing 0.05% (v/v) Tween 20 for 30 minutes at RT. After washing with tris-buffered saline, membranes were incubated with first antibodies (anti-phospho-p44/42 MAPK (Thr202/Tyr204; 1:1,000), anti-p44/42 MAPK (1:1,000), both obtained from Cell Signaling Technology (Frankfurt am Main, Germany)) overnight at RT. After rinsing with tris-buffered saline, membranes were decorated with the second antibody (anti-rabbit-horseradish peroxidase, 1:3,000, Amersham Pharmacia Biotech, Freiburg, Germany) for 1.5 hours at RT. Immunoreactive bands were visualized with the Western Lightning chemiluminescence reagent (PerkinElmer Life Sciences, Rodgau-Jügesheim, Germany) and exposure to Hyperfilm (Amersham Pharmacia Biotech, Freiburg, Germany).

TGF-β1 ELISA

HaCaT cells were seeded in six-well plates and grown to confluency. After 24 hours in the presence of fetal calf serum-free medium, cells were stimulated with *trans*-cinnamoyl-OLRGIL-NH_2 (10^{-4} M), tc-AP (10^{-4} M), or trypsin (10^{-8} M) in the presence or absence of 10 μM PD98059. Media were collected on ice after 96 hours and centrifuged for 5 minutes at 1,500 r.p.m. (4°C) in a Heraeus Megafuge 1.0R (Kendro, Osterode, Germany). Cells were harvested by trypsinization and counted. ELISA was carried out according to the manufacturer's instructions (R&D Systems GmbH, Wiesbaden, Germany).

Statistics

All results are expressed as means ± SEM for a series of experiments. Differences between data were tested by Student's *t*-tests for unpaired data. $P \leqslant 0.05$ was considered statistically significant.

CONFLICT OF INTEREST

These authors state no conflict of interest.

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